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Effect of Chlorine Treatment on Infectivity of Hepatitis A Virus

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16. ABSTRACT

This study examined the effect of chlorine treatment on the infectivity of heptatis A virus (HAV). Prodromal chimpanzee feces, shown to induce hepatitis in marmosets (Saguinus sp.), was clarified, and the virus was precipitated with 7% polyethylene glycol 6000, harvested and resuspended. The suspension was layered onto 5 to 30% linear sucrose gradients and centrifuged; the fractions containing HAV were dialyzed, and a 1:500,000 dilution of this preparation induced hepatitis and seroconversion in 2 of 4 marmosets. A 1:50 dilution of this preparation served as inoculum. Untreated inoculum induced overt hepatitis and seroconversion in 100% (5 of 5) of marmosets inoculated intramuscularly. Inoculum treated for various periods (15, 30, or 60 min) with 0.5, 1.0 or 1.5 mg of free residual chlorine per liter induced hepatitis in 14% (2 of 14), 8% (1 of 12), and 10% (1 of 10) of marmosets, respectively, and induced seroconversion in 29, 33, and 10% of the animals. Inoculum treated with 2.0 or 2.5 mg of free residual chlorine per liter was not infectious in marmosets as determined by absence of hepatitis and seroconversion in the 13 animals tested. Thus, treatment levels of 0.5 to 1.5 mg of free residual chlorine per liter inactivated most but not all HAV in the preparation, whereas concentrations of 2.0 and 2.5 mg of free residual chlorine per liter destroyed the infectivity completely. These results suggest that HAV is somewhat more resistant to chlorine than are other enteroviruses.

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JOURNAL ARTICLE:

Effect of Chlorine Treatment on Infectivity of Hepatitis A Virus

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This study examined the effect of chlorine treatment on the infectivity of hepatitis A virus (HAV). Prodromal chimpanzee feces, shown to induce hepatitis in marmosets (*Saguinus sp.*), was clarified, and the virus was precipitated with 7% polyethylene glycol 6000, harvested, and resuspended. The suspension was layered onto 5 to 30% linear sucrose gradients and centrifuged; the fractions containing HAV were dialyzed, and a 1:500,000 dilution of this preparation induced hepatitis and seroconversion in 2 of 4 marmosets. A 1:50 dilution of this preparation served as inoculum. Untreated inoculum induced overt hepatitis and seroconversion in 100% (5 of 5) of marmosets inoculated intramuscularly. Inoculum treated for various periods (15, 30, or 60 min) with 0.5, 1.0, or 1.5 mg of free residual chlorine per liter induced hepatitis in 14% (2 of 14), 8% (1 of 12), and 10% (1 of 10) of marmosets, respectively, and induced seroconversion in 29, 33, and 10% of the animals. Inoculum treated with 2.0 or 2.5 mg of free residual chlorine per liter was not infectious in marmosets as determined by absence of hepatitis and seroconversion in the 13 animals tested. Thus, treatment levels of 0.5 to 1.5 mg of free residual chlorine per liter inactivated most but not all HAV in the preparation, whereas concentrations of 2.0 and 2.5 mg of free residual chlorine per liter destroyed the infectivity completely. These results suggest that HAV is somewhat more resistant to chlorine than are other enteroviruses.

In the United States, viral hepatitis ranks fourth among reportable infectious diseases and is the most common, serious infectious disease for which no specific treatment exists. Viral hepatitis is caused by two distinctly different viruses, hepatitis A virus (HAV) and hepatitis B virus. From 1972 to 1981, about 40,000 to 50,000 cases of hepatitis A and 6,000 to 9,000 cases of hepatitis B have been reported annually in the United States, but the actual incidence of clinical disease and subclinical infection is undoubtedly several times greater than the number of reported cases.

The fecal-oral transmission of HAV by contamination of water supplies, food, and drink is well documented by the many detailed reports (3, 9, 15) of hepatitis A epidemics. Chlorine (hypochlorous acid [HOCl]), generally accepted as a universal water disinfectant, has been shown to be an effective virucidal agent against adenovirus and various enteroviruses (5, 6, 10, 13, 14, 17). Although the effect of chlorine on HAV has not been extensively examined, Neefe et al. (10) showed several years ago that large amounts of chlorine destroyed infectivity of HAV in crude fecal filtrates.

The virological, serological, and pathological findings that characterize HAV infections and type A hepatitis in human patients can be reproduced experimentally in marmoset monkeys. This report describes the results of a study to determine the effect of chlorine treatment on the infectivity in marmosets of HAV derived from fecal material.

MATERIALS AND METHODS

Animals. Adult wild-caught and colony-born and -reared white-lipped marmosets (*Saguinus fuscicollis, S. nigricollis*) and red-bellied marmosets (*S. labiatus labiatus*) were used. Marmosets were maintained by procedures used routinely in the management of the marmoset colony located at Rush-Presbyterian-St. Luke's Medical Center (11, 16). Animals were divided into experimental groups of four to six animals per group, and base-line evaluations of serum transaminase levels and liver histology were performed.

HAV source material. In previous work, a chimpanzee (Ch. 122) was inoculated with human acute-phase hepatitis feces (GBG-BM isolate) and developed acute

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hepatitis at 21 days post inoculation (dpi). Stool specimens were obtained daily and frozen at −70°C. Examination of these stool specimens for HAV antigen (HAV Ag) by radioimmunoassay (RIA) and for virus particles by electron microscopy revealed high levels of HAV in feces obtained at 11 to 18 dpi. A 20% fecal pool prepared from stools collected 13, 14, and 15 dpi induced hepatitis in five of six and seroconverted six of six marmosets inoculated. This pool served as the HAV source for this study.

Sera. Normal human and marmoset sera, lacking anti-HAV, were used as diluent and control sera as needed. Anti-HAV sera, obtained from convalescent humans or marmosets and containing high titers of anti-HAV (greater than or equal to 1:20,000 for human sera; greater than or equal to 1:5,000 for marmoset sera), were used to assay for HAV Ag. Partially purified gamma globulin was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography (DE 52, Whatman Ltd., England) and was labeled with ^{125}I for RIA (4).

Partial purification of HAV. HAV was partially purified from chimpanzee (prodromal phase) feces by the method of Moritsugu et al. (7). Feces (235 g) were added to a 500-ml polycarbonate screw cap bottle containing 30 g of glass beads (4 mm diameter) and 250 ml of 0.01 M Tris buffer (pH 8.0) and shaken vigorously for several minutes. HAV was recovered from the supernatant after clarification (2,700 rpm, 45 min, 4°C); the amount of supernatant withdrawn was replaced by fresh buffer, and the extraction was repeated. The pooled supernatant obtained after four extractions (approximately 500 ml) was clarified in a JA21 rotor (10,000 rpm, 60 min, 5°C), and the resulting supernatant was designated C-1 and stored at −70°C. C-1 preparation (30 ml) was thawed and made up to 7% polyethylene glycol 6000; the solution was mixed periodically and incubated at 4°C overnight. The precipitate was harvested by centrifugation in a JA20 rotor (8,000 rpm, 5°C, 30 min) and resuspended in 0.01 M Tris buffer (pH 8.0). This material was separated on a linear sucrose gradient (5 to 30% in 0.01 M Tris buffer, pH 8.0, with 0.1% Nonidet P-40). After centrifugation in a SW27 rotor (26,000 rpm, 180 min, 5°C), the gradients were fractionated, and fractions were examined by RIA for HAV Ag. Fractions which contained HAV were pooled, and the virus was sedimented in a SW41 rotor (38,000 rpm, 120 min, 5°C) and resuspended in 0.05 M phosphate buffer.

Chlorine inactivation experiments. The glassware was thoroughly cleaned and rinsed with glass-distilled, deionized water. Stock solutions of sodium hypochlorite were prepared from Clorox (Clorox Corp., Oakland, Calif.). Chlorinated water was prepared by adding 5 mg of a hypochlorite solution (prepared from Clorox) per liter to glass-distilled, deionized water. The chlorinated water was allowed to stand at room temperature for 1 week before use. All buffers were prepared with chlorinated water to produce chlorine demand-free buffers (CDF buffers). The buffer system for the inactivation experiments consisted of a 0.05 M phosphate buffer (KH_2PO_4–K_2HPO_4) prepared at pH 7. Buffers were exposed to UV light for 48 to 72 h and then were tested for chlorine by the orthotolidine test (1). Buffers were considered chlorine demand free and suitable for use when the orthotolidine test was negative.

Chlorine determinations. The free residual chlorine content of all stock and treatment solutions was determined by amperometric titration (1). The chlorine residual of treatment and control solutions immediately before and after each treatment experiment was determined by the *N*, *N*, diethyl-*p*-phenyldiamine (DPD) colorimetric method (1).

Neutralizer solution. To neutralize the effect of chlorine, a 10% solution of sodium thiosulfate ($Na_2S_2O_3$) in 0.001 M phosphate buffer (KH_2PO_4–Na_2HPO_4) (pH 7.4) was prepared, filtered (0.22-nm membrane), and stored at 4°C. The solution was autoclaved on the day of the experiments.

Chlorine treatment conditions. The chlorine demand of the HAV solution was determined by both amperometric and DPD methods to be 0.1 to 0.2 mg of HOCl per liter. Treatment solutions were prepared at pH 7.0 with the desired free residual chlorine (0.5, 1.0, 1.5, 2.0, and 2.5 mg per liter) plus 0.2 mg/l to satisfy the demand of the HAV solution. HAV solutions were treated for 15, 30, or 60 min at 5°C with gentle agitation in an oscillating water bath.

Chlorine treatment procedure. A scaled-down system similar to that of Scarpino et al. (13) was used to conserve the volume of infectious HAV needed. On the day of each experiment, sufficient chlorine was added to CDF buffer to give the desired chlorine concentration. Samples of the CDF buffer solution having the desired chlorine residual were placed in beakers labeled "test," "chlorine control" and "temperature control". Another beaker was labeled "virus control" and was filled with an equal volume of the CDF buffer without chlorine added. The four beakers were allowed to "soak" at room temperature for 1 h to eliminate any chlorine demand associated with the beakers. After "soaking," the contents of each beaker were discarded, replaced with a similar volume of CDF buffer solution, with or without chlorine, placed into an oscillating 5°C water bath, and equilibrated for 30 min until the desired temperature was reached. At the beginning and end of each inactivation experiment, 10 ml was removed from the "chlorine control" beaker, and the residual chlorine was determined. Partially purified HAV was added to the "virus control" and "test" beakers, which were agitated constantly throughout the contact period. At the end of the contact period, 5 ml was removed from the "virus control" and "test" beakers and diluted (1:2) with demand-free water, and the residual chlorine was determined. The reaction was stopped by the addition of 0.1 ml of sodium thiosulfate neutralizing solution immediately after removal of the 5 ml for the chlorine determination. The remaining reaction mixtures were evaluated in vivo as described below.

RIA. HAV Ag was detected by a microtiter solid-phase RIA as previously described (12). Antibodies to HAV were detected by RIA, using the HAVAB test kits (Abbott Laboratories, Inc., North Chicago, Ill.) following the manufacturer's procedure.

In vivo assay of infectivity. Infectivity of a partially purified HAV preparation was confirmed before the effect of chlorine on HAV was examined. Treatment groups consisted of four to six marmosets inoculated intramuscularly with 1 ml of chlorine-treated HAV suspension, and control groups consisted of two to five marmosets inoculated intramuscularly with an equivalent volume of untreated HAV suspension. De-

velopment of infection and induction of hepatitis were monitored by weekly serum transaminase levels, evaluation of liver biopsies obtained at 2-week intervals, and development of antibodies to HAV (anti-HAV) as previously described (12). The observation period was 120 days for animals inoculated with untreated preparations and 150 days for those inoculated with HOCl-treated preparations. Animals receiving chlorine-treated inoculum were monitored for an additional 30 days, since partial inactivation of HAV could result in extended incubation periods or inapparent disease.

RESULTS

Infectivity of partially purified HAV. Infectivity of the polyethylene glycol-sucrose preparation of HAV was evaluated in marmosets before initiation of the treatment experiments. A 1:5, 1:10, or 1:20 dilution of the HAV preparation induced hepatitis in three of four, two of four, and two of three animals, respectively. For the seven marmosets which developed hepatitis, incubation periods were 28 to 30 dpi for six animals and 42 dpi for one animal. The infectivity of the preparation, as reflected by induction of seroconversion in 100% of the animals (11 of 11) and hepatitis in 64% (7 of 11), indicated that the titer was sufficient for the preparation to be used in the chlorine treatment experiments. The titer of the preparation was determined from data obtained by using successive 10-fold dilutions of the preparation (1:50 to 1:500,000) inoculated as control groups along with the chlorine treatment experiments. The results of the titration are shown in Table 1. The undiluted partially purified HAV preparation had $10^{-5.08}$ 50% marmoset infective doses per ml (MID_{50}/ml). Thus, the 1:50 dilution used in the chlorine treatment experiments had a titer of $10^{-3.38}$ MID_{50}/ml, or approximately 1,500 MID_{50}/ml.

In vivo evaluation of chlorine-treated HAV. A 1:50 dilution of the partially purified polyethylene glycol-sucrose preparation of HAV was selected for the chlorine treatments. The chlorine demand of this preparation was 0.13 mg of free residual chlorine per liter (average of 5 determinations, range 0.09 to 0.16 mg/liter) by amperometric method and 0.21 mg/liter (average of 10 determinations, range 0.10 to 0.38 mg/liter) by DPD method. Therefore, chlorine treatment solutions were prepared with 0.2 mg of free residual chlorine per liter more than the desired treatment levels to insure that the free residual desired was present after the chlorine demand of the virus preparation had been satisfied.

Partially purified HAV was diluted 1:50 in CDF buffer and treated for various periods (15, 30, or 60 min) with 0.5, 1.0, 1.5, 2.0, or 2.5 mg of free residual chlorine per liter at 5°C and pH 7.0 with gentle agitation. After the exposure period, the chlorine was neutralized with sodium thiosulfate, and the treated preparations were inoculated into marmosets. Untreated control HAV preparations were processed in a similar manner and inoculated into marmosets as described above (Table 1).

TABLE 1. Titration of partially purified HAV in marmoset monkeys (*Saguinus* sp.)

Inoculum dilution[a]	No. developing hepatitis/no. inoculated	No. that seroconverted/ no. inoculated
1:50	5/5	5/5
1:500	3/3	3/3
1:5,000	3/4	4/4
1:50,000	3/5	4/5
1:500,000	2/4	2/4

[a] Dilutions were made in CDF buffer.

The results of 11 treatment experiments and 2 control experiments are shown in Table 2. Treatment of HAV with 0.5 mg of free residual chlorine per liter in three experiments (15-, 30-, and 30-min exposure) resulted in decreased infectivity: 14% (2 of 14) of the animals developed hepatitis (versus 100% of the controls), and 29% (4 of 14) developed antibodies to HAV (versus 100% of the controls). In addition, the incubation period (71 days) and seroconversion time (85 days) were longer than those of the controls (40 and 36 days, respectively). HAV treated with 1 mg/liter in three experiments (30-, 30-, and 60-min exposure) induced hepatitis in 8% (1 of 12) of the animals, and 33% (4 of 12) seroconverted. Treatment with 1.5 mg/liter in two experiments (30-min exposure) resulted in 10% (1 of 10) of the animals developing hepatitis and seroconverting. Treatment of HAV with 2.0 mg (two experiments with 30-min exposure) or 2.5 mg (one experiment with 30-min exposure) of free residual chlorine prevented the development of apparent hepatitis and antibodies to HAV.

Immunogenic effect of chlorine-treated HAV. Six animals which had been previously inoculated with chlorine-treated HAV (four with 2.0 mg/liter and two with 2.5 mg/liter) were given two additional inoculations (at 14-day intervals) of HAV treated with 2.5 mg of HOCl per liter (30 min). None of these animals developed detectable anti-HAV antibody.

DISCUSSION

The 1:50 dilution of HAV preparation used for all treatment experiments contained on the order of 1,500 MID_{50}/ml. In the 0.5 mg/liter treatment experiments, only 14% (2 of 14) of the animals developed hepatitis, and 29% (4 of 14) developed antibodies. Thus, the incidence of both overt hepatitis and seroconversion was well below the

TABLE 2. Infectivity of chlorine-treated partially purified HAV in marmoset monkeys (*Saguinus* sp.)[a]

Treatment		No. developing hepatitis/no. inoculated	% Developing hepatitis	Mean incubation period (days)	No. that seroconverted/ no. inoculated	% That seroconverted	Mean seroconversion time (days)
Mg of HOCl per liter	Min						
0	30	3/3	100	37	3/3	100	28
	30	2/2		45	2/2		48
0.5	15	1/5	14	71	2/5	29	85
	30	1/5		71	2/5		85
	30	0/4		—	0/4		—
1.0	30	0/4	8	—	2/4	33	107
	30	1/4		—	1/4		48
	60	0/4		—	1/4		75
1.5	30	1/5	10	70	1/5	10	70
	30	0/5		—	0/5		—
2.0	30	0/4	0	—	0/4	0	—
	30	0/4		—	0/4		—
2.5	30	0/5	0	—	0/5	0	—

[a] HAV virus was diluted 1:50 with treatment buffer, pH 7.0, containing the desired level of HOCl at 5°C and treated for the indicated time with gentle agitation.

50% level, implying that the titer, after treatment, was less than 1 MID_{50}/ml or a minimal reduction in titer of $10^{-3.38}$ MID_{50}/ml. The extended incubation and seroconversion periods observed in the treated groups are also indicative of low infective doses and have been seen repeatedly in titration experiments in marmosets. The protracted seroconversion periods (70 to 107 days) in the 0.5- to 1.5-mg free residual chlorine treatment groups were interpreted as a true reflection of infection and multiplication of HAV with subsequent development of anti-HAV, since immunization of animals three times with HAV treated with 2.0 or 2.5 mg of free residual chlorine per liter failed to induce antibodies recognized as anti-HAV.

The development of overt hepatitis in 1 of 10 animals which received virus treated with 1.5 mg/liter for 30 min was probably due to the presence of small aggregates of HAV which were not completely penetrated by the chlorine. The fact that only this animal seroconverted suggests that the vast majority of single or small aggregates of virus were inactivated by 1.5 mg of free residual chlorine per liter.

The decreased incidence of disease and development of anti-HAV, and the lengthening of the incubation period and appearance time of anti-HAV, indicated that treatment levels of 0.5 to 1.5 mg/liter for 30 min have inactivated most, but not all, HAV in the preparations. Concentrations of 2.0 or 2.5 mg/liter destroyed the infectivity completely. However, the levels of free residual chlorine required to inactivate HAV within the environment, protected by fecal material aggregated and dispersed in water, are unknown.

An American Water Works Association Committee (2) recently recommended that appropriate disinfection of drinking water to ensure viral inactivation could be achieved by maintaining a free chlorine residual of 1.0 mg of HOCl per liter for at least 30 min at water pH values of less than 8.0. Data from a number of investigations reviewed by the National Academy of Sciences (8) indicated that under these conditions, enterovirus numbers were consistently reduced by two orders of magnitude in less than 5 min. The results of this investigation suggest that HAV is somewhat more resistant to chlorine than are other enteroviruses.

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